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(54) Title: NOVEL TNFSF13B PROTEIN VARIANTS

(57) Abstract: The present invention provides novel proteins that are variants derived from human TNFSF13b. The novel proteins of the invention are useful in one embodiment for providing TNFSF13b activity in a human subject suffering from a disorder associated with insufficient or absent TNFSF13b activity.

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## NOVEL hTNFSF13b PROTEIN VARIANTS

### FIELD OF THE INVENTION

The present invention relates to novel polypeptides, designated herein as hTNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9, and therapeutic utility of these polypeptides.

### BACKGROUND OF THE INVENTION

The TNF family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including proliferation, cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. One member of the TNF family is TNFSF13b. TNFSF13b is the official name adopted by the TNF Congress for BLyS, TALL-1, BAFF, THANK, neutrokine- $\alpha$ , and zTNF (for review see Locksley et al. Cell 2001 104:487). Human TNFSF13b (hTNFSF13b) is a 285-amino acid type II membrane-bound protein that possesses a N-terminal cleavage site that allows for the existence of both soluble and membrane bound proteins. Functionally, TNFSF13b appears to regulate B cell and some T cell immune responses.

Although international patent application publication WO98/18921 discloses hTNFSF13b, that application does not describe specific variants of hTNFSF13b. The present application specifically discloses therapeutically useful variants of hTNFSF13b.

### SUMMARY OF THE INVENTION

The present invention provides isolated human TNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, and variant 9 polypeptides as described herein.

The present invention also provides an isolated TNFSF13b variant wherein the polypeptide further comprises at least one specified substitution, insertion, or deletion corresponding to portions or specific residues of TNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, and variant 9.

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A polypeptide of the present invention includes an isolated TNFSF13b variant polypeptide comprising at least one fragment, domain, or specified variant of at least 90-100% of the contiguous amino acids of at least one portion of TNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, and variant 9.

The present invention also provides compositions, including pharmaceutical compositions, comprising a TNFSF13b variant polypeptide or an anti-TNFSF13b variant polypeptide antibody, wherein the composition has a measurable effect on an activity associated with a particular TNFSF13b variant polypeptide as disclosed herein.

A method of treatment or prophylaxis based on a TNFSF13b variant polypeptide associated activity as disclosed herein can be effected by administration of one or more of the polypeptides, antibodies, and/or compositions described herein to a mammal in need of such treatment or prophylactic. Accordingly, the present invention also includes methods for the prophylaxis or treatment of a patho-physiological condition in which at least one cell type involved in said condition is sensitive or responsive to a TNFSF13b variant polypeptide, TNFSF13b variant polypeptide-encoding polynucleotide, TNFSF13b variant nucleic acid, TNFSF13b variant polypeptide antibody, host cell, transgenic cell, and/or composition of the present invention.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The polynucleotides and polypeptides of the present invention are derived from native, human TNFSF13b (hTNFSF13b) polynucleotides and polypeptides. Unless specified otherwise, the term "TNFSF13b" as used herein refers to human TNFSF13b. Accordingly, polynucleotides and polypeptides of the present invention are designated as "TNFSF13b variant polynucleotides" or "TNFSF13b variant polypeptide-encoding polynucleotides" and "TNFSF13b variant polypeptides." For purposes of convenience, variants of the soluble form of TFSF13b are generally designated herein by the term "variant". In particular, when immediately followed by an numerical designation (e.g., variant 1), the term "variant" refers to a specific molecule or group of molecules as defined herein. A complete designation wherein the term "variant" is immediately followed by a numerical designation and a molecule type (e.g., variant 1 polypeptide)

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refers to a specific type of molecule within the designated group of molecules as designated herein.

The terms "variant polypeptide-encoding polynucleotides" or "variant polynucleotides" and "variant polypeptides" wherein the term "variant" is followed by an actual numerical designation as used herein encompass novel polynucleotides and polypeptides, respectively, which are further defined herein. The variant molecules described herein may be isolated or prepared from a variety of sources including, but not limited to, preparation by recombinant or synthetic methods.

One aspect of the present invention provides an isolated nucleic acid molecule comprising a polynucleotide which encodes a variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9 polypeptide or fragment and/or derivative thereof as defined herein.

"TNFSF13b variant polypeptide derivative" is intended to refer to an "active" TNFSF13b polypeptide, wherein activity is as defined herein, having at least about 90% amino acid sequence identity with a TNFSF13b variant polypeptide having a deduced amino acid sequences as described herein. Such TNFSF13b polypeptide derivatives include, for instance, derivatives of TNFSF13b variant polypeptides, wherein one or more amino acid residues are added, substituted or deleted, at the N- or C-terminus or within the sequences shown. Ordinarily, a derivative of a TNFSF13b variant polypeptide will have at least about 90% amino acid sequence identity, preferably at least about 91% sequence identity, yet more preferably at least about 92% sequence identity, yet more preferably at least about 93% sequence identity, yet more preferably at least about 94% sequence identity, yet more preferably at least about 95% sequence identity, yet more preferably at least about 96% sequence identity, yet more preferably at least about 97% sequence identity, yet more preferably at least about 98% sequence identity, yet more preferably at least about 99% amino acid sequence identity with the amino acid sequence described, with or without the signal peptide.

The term "homolog" refers to a molecule from a given organism that exhibits sequence similarity and/or identity and has substantially similar biological activity as a molecule from a different organism. For example, a mouse (murine) polypeptide that functions in the mouse in a manner equivalent to which human TNFSF13b functions in humans may be referred to as a mouse or murine homolog of TNFSF13b, as well as

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mouse or murine TNFSF13b. The term "homolog" is also intended to encompass two or more genes or proteins from different organisms or within a single organism that exhibit sequence similarity and/or identity. The term "homolog" as used herein includes allelic variants, as well as splice variants from a TNFSF13b polynucleotide sequence.

"Isolated," when used to describe the various polypeptides disclosed herein, means a polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Preferably, the isolated polypeptide is free of association with all components with which it is naturally associated. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the TNFSF13b variant polypeptide natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

In referring to amino acid sequences, the term "similar" or "similarity" describes amino acid residues which are either identical between different amino acid sequences; or represent conservative amino acid substitutions between different sequences.

Conservative amino acid substitutions are listed in Table 1 and discussed infra. The term "identity" describes amino acid residues which are identical between different amino acid sequences. Amino acid sequence similarity or identity with respect to each TNFSF13b variant amino acid sequence identified herein is defined as the percentage of amino acid residues in a candidate sequence that are similar or identical with the amino acid residues in a TNFSF13b variant polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence similarity or identity.

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Table 1. Conservative Substitutions

Residue	Substitutions	Preferred Substitution
Ala (A)	gly, val, leu, ile, ser, met, thr	val
Arg (R)	lys, gln, asn, his	lys
Asn (N)	gln	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	ala, ile, leu, pro, ser, met, val	ala
His (H)	asn, gln, lys, arg	arg
Ile (I)	leu, val, met, ala, phe, norleucine	leu
Leu (L)	norleucine, ile, val, met, ala phe	ile
Lys (K)	arg, gln, asn, his	arg
Met (M)	ala, gly, ile, leu, phe, ser, val	leu
Phe (F)	leu, val, ile, ala, trp, tyr	tyr
Pro (P)		
Ser (S)	ala, gly, ile, leu, met, thr, val	thr
Thr (T)	ala, gly, ile, leu, met, ser, val	ser
Trp (W)	tyr, phe	tyr
Tyr (Y)	trp, phe, thr, ser	phe
Val (V)	ala, ile, leu, met, ser, met, gly, norleu	leu

"Percent (%) amino acid sequence identity" with respect to the TNFSF13b variant amino acid sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a TNFSF13b variant polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN, ALIGN-2, Megalign (DNASTAR) or BLAST (e.g., Blast, Blast-2, WU-Blast-2) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For example, the percent identity values used herein are generated using WU-BLAST-2 [Altschul, *et al.*, *Methods in Enzymology* 266:460-80 (1996)]. Most of the WU-BLAST-2 search parameters are set to the default values. Those not set to default values, i.e., the adjustable parameters, are set with the following values: overlap span = 1; overlap

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fraction = 0.125; word threshold (T) = 11; and scoring matrix = BLOSUM 62. For purposes herein, a percent amino acid sequence identity value is determined by dividing (a) the number of matching identical amino acid residues between the amino acid sequence of the TNFSF13b variant polypeptide of interest and the comparison amino acid sequence of interest (i.e., the sequence against which the TNFSF13b variant polypeptide of interest is being compared) as determined by WU-BLAST-2, by (b) the total number of amino acid residues of the TNFSF13b variant polypeptide of interest, respectively.

The term "amino acid" is used herein in its broadest sense, and includes naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs and derivatives. The latter includes molecules containing an amino acid moiety. One skilled in the art will recognize, in view of this broad definition, that reference herein to an amino acid includes, for example, naturally occurring proteogenic L-amino acids; D-amino acids; chemically modified amino acids such as amino acid analogs and derivatives; naturally-occurring non-proteogenic amino acids such as norleucine, beta-alanine, ornithine, etc.; and chemically synthesized compounds having properties known in the art to be characteristic of amino acids. As used herein, the term "proteogenic" indicates that the amino acid can be incorporated into a peptide, polypeptide, or protein in a cell through a metabolic pathway.

The incorporation of non-natural amino acids, including synthetic non-native amino acids, substituted amino acids, or one or more D-amino acids into the TNFSF13b variant peptides, polypeptides, or proteins of the present invention ("D-TNFSF13b variant polypeptides") is advantageous in a number of different ways. D-amino acid-containing peptides, polypeptides, or proteins exhibit increased stability *in vitro* or *in vivo* compared to L-amino acid-containing counterparts. Thus, the construction of peptides, polypeptides, or proteins incorporating D-amino acids can be particularly useful when greater intracellular stability is desired or required. More specifically, D-peptides, polypeptides, or proteins are resistant to endogenous peptidases and proteases, thereby providing improved bioavailability of the molecule and prolonged lifetimes *in vivo* when such properties are desirable. When it is desirable to allow the peptide, polypeptide, or protein to remain active for only a short period of time, the use of L-amino acids therein will permit endogenous peptidases, proteases, etc., in a cell to digest the molecule *in vivo*, thereby limiting the cell's exposure to the molecule. Additionally, D-peptides,

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polypeptides, or proteins cannot be processed efficiently for major histocompatibility complex class II-restricted presentation to T helper cells, and are therefore less likely to induce humoral immune responses in the whole organism.

In addition to using D-amino acids, those of ordinary skill in the art are aware that modifications in the amino acid sequence of a peptide, polypeptide, or protein can result in equivalent, or possibly improved, second generation peptides, polypeptides, or proteins, that display equivalent or superior functional characteristics when compared to the original amino acid sequences. Alterations in the TNFSF13b variant peptides, polypeptides, or proteins of the present invention can include one or more amino acid insertions, deletions, substitutions, truncations, fusions, shuffling of subunit sequences, and the like, either from natural mutations or human manipulation, provided that the sequences produced by such modifications have substantially the same (or improved or reduced, as may be desirable) activity(ies) as the naturally-occurring counterpart sequences disclosed herein.

Amino acid substitutions in the TNFSF13b variant polypeptides of the present invention can be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, etc. As is known to one of skill in the art, amino acids generally can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Exemplary substitutions that take various of the foregoing characteristics into consideration in order to produce conservative amino acid changes resulting in silent changes within the present peptides, polypeptides, or proteins can be selected from other members of the class to which the naturally occurring amino acid belongs.

Biologically functional equivalents derived from the present TNFSF13b variant polypeptides can have any number of conservative or non-conservative amino acid changes that do not significantly affect their activity(ies), or that increase or decrease activity as desired. Preferably, the number of changes in derivatives of TNFSF13b variant polypeptides may be 10, 6, 5, 3 or fewer changes, or any value therein. The nucleotide sequences encoding the polypeptide derivatives will contain corresponding base substitutions to code for the expression of TNFSF13b derivatives. In any case, derivatives of TNFSF13b variant polypeptides exhibit the same or similar biological or

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immunological activity(ies) as that(those) of the TNFSF13b variant polypeptides specifically disclosed herein, or increased or reduced activity, if desired. The activity(ies) of the biologically functional equivalents of TNFSF13b variant polypeptides can be determined by the methods described herein. Derivatives of TNFSF13b variant polypeptides have activity(ies) differing from those of the presently disclosed molecules by about  $\pm$  50% or less, preferably by about  $\pm$  40% or less, more preferably by about  $\pm$  30% or less, more preferably by about  $\pm$  20% or less, and even more preferably by about  $\pm$  10% or less, when assayed by the methods disclosed herein.

The term "epitope tagged" where used herein refers to a chimeric polypeptide comprising a TNFSF13b variant polypeptide, or domain sequence thereof, fused to a "tag polypeptide." The tag polypeptide has enough residues to provide an epitope against which an antibody may be made, or which can be identified by some other agent, yet is short enough such that it does not interfere with the activity of the TNFSF13b variant polypeptide. Suitable epitope tag polypeptides that are well known to those of skill in the art include the hexahistidine His tag and the eight amino acid FLAG tag.

"Active" or "activity" for the purposes herein refers to form(s) of TNFSF13b variant polypeptide which retain all or a portion of the biologic and/or immunologic activities of native or naturally-occurring TNFSF13b polypeptide. Elaborating further, "biological" activity refers to a biological function (either inhibitory or stimulatory) caused by a native or naturally-occurring TNFSF13b polypeptide other than the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring TNFSF13b polypeptide. An "immunological" activity refers only to the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring TNFSF13b polypeptide.

"Antibodies" (Abs) are glycoproteins which exhibit binding specificity to a specific antigen. The term "antibody" is used in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody

fragments include Fab, Fab', F(ab')<sub>2</sub> and Fv fragments; diabodies; linear antibodies [Zapata, et al., *Protein Engin.* 8 (10):1057-62 (1995)]; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

“Single-chain Fv” or “sFv” antibody fragments comprise the V<sub>H</sub> and V<sub>L</sub> domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V<sub>H</sub> and V<sub>L</sub> domain, which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun, *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore, eds., Springer-Verlag, New York, pp. 269-315 (1994).

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody’s natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

An “TNFSF13b variant antibody” or “TNFSF13b variant antibody” refers to an antibody as defined herein that recognizes and binds at least one epitope of a TNFSF13b variant polypeptide of the present invention. The term “TNFSF13b variant polypeptide antibody” or “TNFSF13b variant antibody” wherein the term “variant” is followed by a numerical designation refers to an antibody that recognizes and binds to at least one epitope of that particular TNFSF13b variant polypeptide as disclosed herein.

The terms “treating,” “treatment,” and “therapy” as used herein refer to curative therapy, prophylactic therapy, and preventive therapy. An example of “preventive therapy” is the prevention or lessened targeted pathological condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

“Chronic” administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. “Intermittent” administration is treatment that is not consecutively done without interruption but, rather, is cyclic in nature.

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Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A "therapeutically-effective amount" is the minimal amount of active agent (e.g., a TNFSF13b variant polypeptide, antagonist or agonist thereof) which is necessary to impart therapeutic benefit to a mammal. For example, a "therapeutically-effective amount" to a mammal suffering or prone to suffering or to prevent it from suffering is such an amount which induces, ameliorates, or otherwise causes an improvement in the pathological symptoms, disease progression, physiological conditions associated with or resistance to succumbing to the afore described disorder.

"Carriers" as used herein include pharmaceutically-acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically-acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecule weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN<sup>®</sup>, polyethylene glycol (PEG), and PLURONIC<sup>®</sup>.

The term "modulate" means to affect (e.g., either upregulate, downregulate or otherwise control) the level of a signaling pathway. Cellular processes under the control of signal transduction include, but are not limited to, transcription of specific genes, normal cellular functions, such as metabolism, proliferation, differentiation, adhesion, apoptosis and survival, as well as abnormal processes, such as transformation, blocking of differentiation and metastasis.

The present invention includes variations in the full-length sequence TNFSF13b variant polypeptide or in various domains of the TNFSF13b variant polypeptides described herein, such as amino acid substitution. Such variations in polypeptides can be introduced, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent 5,364,934.

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TNFSF13b variant polypeptide fragments are also provided herein. Such fragments may be truncated at the N-terminus or C-terminus, or may lack internal residues, for example, when compared with a full length or native protein. Certain fragments contemplated by the present invention may lack amino acid residues that are not essential for a desired biological activity of the TNFSF13b variant polypeptide. TNFSF13b variant polypeptide fragments may be prepared by any of a number of conventional techniques known in the art.

Covalent modifications of TNFSF13b variant polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a TNFSF13b variant polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of a TNFSF13b variant polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking TNFSF13b variant polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-TNFSF13b variant polypeptide antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithiolpropioimidate].

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains [Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the TNFSF13b variant polypeptides included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native TNFSF13b variant polypeptide sequence and/or adding one or more glycosylation sites

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that are not present in the native sequences of TNFSF13b variant polypeptides. Additionally, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

Addition of glycosylation sites to TNFSF13b variant polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequences of TNFSF13b variant polypeptides (for O-linked glycosylation sites). The TNFSF13b variant amino acid sequences may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the TNFSF13b variant polypeptides at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the TNFSF13b variant polypeptides is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of carbohydrate moieties present on the TNFSF13b variant polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Sojar, *et al.*, *Arch. Biochem. Biophys.* 259:52-7 (1987), and by Edge, *et al.*, *Anal. Biochem.* 118:131-7 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura, *et al.*, *Meth. Enzymol.* 138:350-9 (1987).

Another type of covalent modification of TNFSF13b variant comprises linking any one of the TNFSF13b variant polypeptides to one of a variety of non-proteinaceous polymers (e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes) in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, or 4,179,337.

TNFSF13b variant polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising a TNFSF13b variant polypeptide fused to another heterologous polypeptide or amino acid sequence. In one embodiment, such a

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chimeric molecule comprises a fusion of a TNFSF13b variant polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of a variant TNFSF13b polypeptide. The presence of such epitope-tagged forms of a TNFSF13b variant polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables a TNFSF13b variant polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

In an alternative embodiment, the chimeric molecule may comprise a fusion of a TNFSF13b variant polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule. The Ig fusions preferably include the substitution of a soluble transmembrane domain deleted or inactivated form of a TNFSF13b variant polypeptide in place of at least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH2 and CH3 or the hinge, CH1, CH2 and CH3 regions of an IgG1 molecule. The production of immunoglobulin fusions is described in U.S. Patent 5,428,130.

In yet a further embodiment, the TNFSF13b variant polypeptides of the present invention may also be modified in a way to form a chimeric molecule comprising a TNFSF13b variant polypeptide fused to a leucine zipper. Various leucine zipper polypeptides have been described in the art. See, e.g., Landschulz, *et al.*, *Science* 240(4860):1759-64 (1988); WO 94/10308; Hoppe, *et al.*, *FEBS Letters* 344(2-3):191-5 (1994); Abel, *et al.*, *Nature* 341(6237):24-5 (1989). It is believed that use of a leucine zipper fused to a TNFSF13b variant polypeptide may be desirable to assist in dimerizing or trimerizing soluble TNFSF13b variant polypeptide in solution.

TNFSF13b variant polypeptides may be produced by recombinant methods as known in the art. Alternative methods which are also well known in the art, may be employed to prepare TNFSF13b variant polypeptides. For instance, the TNFSF13b variant polypeptide sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, e.g., Stewart, *et al.*, *Solid-Phase Peptide Synthesis*, W.H. Freeman & Co., San Francisco, CA (1969); Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963)]. Various portions of a TNFSF13b variant polypeptide may be

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chemically synthesized separately and combined using chemical or enzymatic methods to produce a full-length TNFSF13b variant polypeptide.

The production of TNFSF13b variant polypeptides by recombinant methods generally entails purifying the polypeptides expressed from cultured cells transformed or transfected with a vector containing a TNFSF13b variant polypeptide-encoding nucleic acid, as is known by one of skill in the art. Host cells are transfected or transformed with expression or cloning vectors for TNFSF13b variant polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation.

TNFSF13b variant polypeptides may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the TNFSF13b variant polypeptide-encoding DNA that is inserted into the vector.

Various forms of a TNFSF13b variant polypeptide may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton X-100<sup>TM</sup>) or by enzymatic cleavage. Cells employed in expression of a TNFSF13b variant polypeptide can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desirable to purify TNFSF13b variant polypeptides from recombinant cell proteins or polypeptides. Various methods of protein purification may be employed and such methods are known in the art and described, for example, in Deutscher, *Methods in Enzymology* 182:83-9 (1990) and Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, NY (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular TNFSF13b variant polypeptide produced.

When the amino acid sequence for a TNFSF13b variant polypeptide encodes a protein which binds to another protein (for example, where the TNFSF13b variant

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polypeptide functions as a receptor), the TNFSF13b variant polypeptide can be used in assays to identify the other proteins or molecules involved in the binding interaction. By such methods, inhibitors of the receptor/ligand binding interaction can be identified. Proteins involved in such binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction. Also, the receptor TNFSF13b variant polypeptide can be used to isolate correlative ligand(s). Screening assays can be designed to find lead compounds that mimic the biological activity of the TNFSF13b variant polypeptides disclosed herein or a receptor for such TNFSF13b variant polypeptides. Typical screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

The present invention further provides anti- TNFSF13b variant polypeptide antibodies. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies. Methods of preparing antibodies are known in the art.

The anti-TNFSF13b variant polypeptide antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR

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regions correspond to those of a non-human immunoglobulin, and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones, *et al.*, *Nature* 321(6069):522-5 (1986); Riechmann, *et al.*, *Nature* 332(6162):323-7 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-6 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones, *et al.*, *Nature* 321(6069):522-5 (1986); Riechmann, *et al.*, *Nature* 332(6162):323-7 (1988); Verhoeyen, *et al.*, *Science* 239(4847):1534-6 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.* 227(2):381-8 (1992); Marks, *et al.*, *J. Mol. Biol.* 222(3):581-97 (1991)]. The techniques of Cole, *et al.*, and Boerner, *et al.*, are also available for the preparation of human monoclonal antibodies (Cole, *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985), and Boerner, *et al.*, *J. Immunol.* 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. This approach is described, for example, in U.S. Patents 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks, *et al.*, *Biotechnology* 10(7):779-83 (1992); Lonberg, *et al.*, *Nature* 368(6474):856-9 (1994); Morrison, *Nature* 368(6474):812-3 (1994);

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Fishwild, *et al.*, *Nature Biotechnology* 14(7):845-51 (1996); Neuberger, *Nature Biotechnology* 14(7):826 (1996); Lonberg and Huszar, *Int. Rev. Immunol.* 13(1):65-93 (1995).

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for a TNFSF13b variant polypeptide, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit. Methods for making bispecific antibodies are known in the art. Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared [Tutt, *et al.*, *J Immunol.* 147(1):60-9 (1991)].

The anti-TNFSF13b variant polypeptide antibodies of the present invention have various utilities. For example, such antibodies may be used in diagnostic assays for TNFSF13b polypeptide expression, e.g., detecting expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, *Monoclonal Antibodies: A Manual of Techniques*, CRC Press, Inc., pp. 147-158 (1987)].

Anti-TNFSF13b variant polypeptide antibodies also are useful for affinity purification from recombinant cell culture or natural sources. In this process, the antibodies are immobilized on a suitable support using methods well known in the art. The immobilized antibody is then contacted with a sample containing the TNFSF13b variant polypeptide to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the TNFSF13b variant polypeptide, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the desired polypeptide from the antibody.

In addition to TNFSF13b variant polypeptide(s), a formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition may comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokines, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitable present

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in combination in amounts that are effective for the purpose intended. The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol), polylactides (U.S. Patent 3,773,919), copolymers of L-glutamic acid gamma-ethyl-L-glutamate, non-degradable ethylene-vinylacetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 degrees C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanisms involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thiosulfide interchange, stabilization may be achieved by modifying sulphhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional pharmaceutically acceptable carriers,

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excipients or stabilizers [*Remington's Pharmaceutical Sciences* 16th edition (1980)], in the form of lyophilized formulations or aqueous solutions.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences, supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, and intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent(s), which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)], polylactides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid.

Microencapsulation of recombinant proteins for sustained release has been successfully performed with human growth hormone (rhGH), interferon, and interleukin-2. Johnson, *et al.*, *Nat. Med.* 2(7):795-9 (1996); Yasuda, *et al.*, *Biomed. Ther.* 27:1221-3 (1993); Hora, *et al.*, *Bio/Technology* 8(8):755-8 (1990); Cleland, "Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems" in

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*Vaccine Design: The Subunit and Adjuvant Approach*, Powell and Newman, Eds., Plenum Press, NY, 1995, pp. 439-462 WO 97/03692; WO 96/40072; WO 96/07399; and U.S. Patent 5,654,010.

The sustained-release formulations of these proteins may be developed using polylactic-coglycolic acid (PLGA) polymer due to its biocompatibility and wide range of biodegradable properties. The degradation products of PLGA, lactic and glycolic acids, can be cleared quickly within the human body. Moreover, the degradability of this polymer can be adjusted from months to years depending on its molecular weight and composition. See Lewis, "Controlled release of bioactive agents from lactide/glycolide polymer" in *Biodegradable Polymers as Drug Delivery Systems* (Marcel Dekker; New York, 1990), M. Chasin and R. Langer (Eds.) pp. 1-41.

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 degrees C, resulting in a loss of biological activity and possible changes in immunogenicity.

It is contemplated that the compounds, including, but not limited to, antibodies, small organic and inorganic molecules, peptides, anti-sense molecules, ribozymes, etc., of the present invention may be used to treat various conditions including those characterized by overexpression and/or activation of the disease-associated genes identified herein. The active agents of the present invention (e.g., antibodies, polypeptides, nucleic acids, ribozymes, small organic or inorganic molecules) are administered to a mammal, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebral, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, intraocular, intralesional, oral, topical, inhalation, pulmonary, and/or through sustained release.

Other therapeutic regimens may be combined with the administration of TNFSF13b variant polypeptide antagonists or antagonists, anti-cancer agents, e.g., antibodies of the instant invention.

For the prevention or treatment of disease, the appropriate dosage of an active agent, (e.g., an antibody, polypeptide, nucleic acid, ribozyme, or small organic or

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inorganic molecule) will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the agent is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, and the discretion of the attending physician. The agent is suitably administered to the patient at one time or over a series of treatments.

Dosages and desired drug concentration of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary artisan. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti and Chappell, "The Use of Interspecies Scaling in Toxicokinetics," in *Toxicokinetics and New Drug Development*, Yacobi, et al., Eds., Pergamon Press, NY, p.4246 (1989).

When *in vivo* administration of a composition comprising a TNFSF13b variant polypeptide, a TNFSF13b variant polypeptide antibody, a TNFSF13b variant polypeptide-encoding nucleic acid, ribozyme, or small organic or inorganic molecule is employed, normal dosage amounts may vary from about 1 ng/kg up to 100 mg/kg of mammal body weight or more per day, preferably about 1 pg/kg/day up to 100 mg/kg of mammal body weight or more per day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Patents 4,657,760, 5,206,344 or 5,225,212. It is within the scope of the invention that different formulations will be effective for different treatment compounds and different disorders, that administration targeting one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue. Moreover, dosages may be administered by one or more separate administrations or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

In another embodiment of the invention, an article of manufacture containing materials useful for the diagnosis or treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable

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containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for diagnosing or treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is typically a TNFSF13b variant polypeptide, antagonist or agonist thereof. The label on, or associated with, the container indicates that the composition is used for diagnosing or treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

#### **hTNFSF13b Variant Polypeptides of the Present Invention**

The human TNFSF13b gene encodes a 285 amino acid protein which is predicted to be a type II membrane bound protein [Moore *et al.*, Science 285:260-263 (1999)]. Sequence analyses suggest the N-terminal 46 amino acids of TNFSF13b are intracellular, followed by a potential transmembrane domain between amino acids 47-72, with the remaining amino acids 73-285 being extracellular. A soluble, 152 amino acid form of TNFSF13b corresponding to amino acids 134-285 of the full-length TNFSF13b polypeptide has been identified from expression of TNFSF13b in mammalian cells. For reference, the soluble form of hTNFSF13b is represented herein by the following amino acid numbering convention:

Human TNFSF13b	1	AVQGPPEETVT	QDCLQLIADS	ETPTIQKGSY	TFVPWLLSFK	40
	41	RGSALLEEKEN	KILVKETGYF	FIYGQVLYTD	KTYAMGHLIQ	80
	81	RKKVHVGDE	LSSLVTLFRCI	QNMPETLPNN	SCYSAGIAKL	120
	121	EEGDELQLAI	PRENAQISLD	GDVTFFGALK	LL	152

Specific amino acid residues of TNFSF13b and variants of the present invention are numbered herein according to this convention for the soluble form of TNFSF13b.

As described in Example 6, a model of human TNFSF13b based on crystal structures of several TNF family members has been used to identify residues of

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TNFSF13b that are likely binding sites for anti-TNFSF13b antibody 4A5-3.1.1-B4 (disclosed in WO 03/016468). These sites in TNFSF13b consist of amino acids which likely reside on the surface of the soluble domain and which potentially form epitopes recognized by anti-TNFSF13b antibodies. Three potential epitopes in soluble TNFSF13b have been identified: 1) K71, T72, Y73, E105; 2) Q26, S29, L139, D140; and 3) L53, K55, E56, K119. The first of these epitopes (K71, T72, Y73, E105) may additionally comprise amino acid residues T69, T106, L107, and/or N109.

The variants of the present invention are derived from TNFSF13b and are based on the three potential epitopes in soluble TNFSF13b described above and in Example 6. The variants of the present invention fall into two general functional classes: 1) TNFSF13b variants which function to induce cell proliferation, with that function being neutralized by an antibody to TNFSF13b (antibody 4A5-3.1.1-B4), and 2) TNFSF13b variants which function to induce cell proliferation, wherein that function is not neutralized by an antibody to TNFSF13b (antibody 4A5-3.1.1-B4). Specifically, variants 1-7 of the present invention fall into the first functional class. Variants 1-7 thereby retain the soluble hTNFSF13b ability to induce B cell proliferation, and like soluble TNFSF, are neutralized by antibody 4A5-3.1.1-B4. In contrast, variants 8-9 fall into the second functional class. Variants 8-9 thus retain the ability of soluble TNFSF13b to induce cell proliferation, but unlike soluble TNFSF13b, are not neutralized by antibody 4A5-3.1.1-B4.

Variants 1-9 of the present invention are derived from TNFSF13b by introducing a substitution at one of the amino acids specified for each of variants 1-9. Generally, any amino acid may be substituted for an amino acid that naturally occurs at a given position in the soluble TNFSF13b polypeptide. Nonetheless, the substituted amino acid preferably will not be proline.

In one embodiment of the present invention, variant 1 is derived from soluble TNFSF13b by introducing a substitution in one or more of the amino acids selected from the group consisting of: threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109. A preferred embodiment of variant 1 is the change E105K, in which the corresponding amino acid from mouse TNFSF13b is substituted for the human amino acid (see Chimera E of Example 6).

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Variant 2 of the present invention is derived from soluble TNFSF13b by introducing a substitution in two or more of the amino acids selected from the group consisting of: threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109.

Variant 3 of the present invention is derived from soluble TNFSF13b by introducing a substitution in three or more of the amino acids selected from the group consisting of: threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109. A preferred embodiment of variant 3 includes the substitutions K71P, T72I, Y73F, in which the corresponding amino acids from mouse TNFSF13b are substituted for the human amino acid (see Chimera B of Example 6).

Variant 4 of the present invention is derived from soluble TNFSF13b by introducing a substitution in four or more of the amino acids selected from the group consisting of: threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109.

Variant 5 of the present invention is derived from soluble TNFSF13b by introducing a substitution in five or more of the amino acids selected from the group consisting of: threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109.

Variant 6 of the present invention is derived from soluble TNFSF13b by introducing a substitution in one or two of the amino acids selected from the group consisting of: glutamine at position 26, serine at position 29, leucine at position 139, and aspartic acid at position 140. A preferred embodiment of variant 6 includes the substitutions L139R, D140N, in which the corresponding amino acids from mouse TNFSF13b are substituted for the human amino acid (see Chimera A of Example 6).

Variant 7 of the present invention is derived from soluble hTNFSF13b by introducing a substitution in one or two of the amino acids selected from the group consisting of: leucine at position 53, lysine at position 55, glutamic acid at position 56,

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and lysine at position 119. A preferred embodiment of variant 7 includes the substitutions L53V, K55R, E56Q, in which the corresponding amino acids from mouse TNFSF13b are substituted for the human amino acid (see Chimera D of Example 6).

Variant 8 of the present invention is derived from soluble hTNFSF13b by introducing a substitution in lysine at position 71, threonine at position 72, tyrosine at position 73, and at least one of the amino acids from the group selected from glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109. The combination of at least 4 substitutions in variant 8 renders a polypeptide that like soluble TNFSF13b induces cell proliferation, but unlike soluble hTNFSF13b, is not neutralized by an antibody (4A5-3.1.1-B4) to TNFSF13b. Variant 8 is exemplified by Chimera C of Example 6.

Variant 9 of the present invention is derived from soluble TNFSF13b by introducing a substitution in threonine at position 69, lysine at position 71, threonine at position 72, tyrosine at position 73, and at least one of the amino acids from the group selected from glutamic acid at position 105, threonine at position 106, leucine at position 107, and asparagine at position 109. The combination of at least 5 substitutions in variant 9 renders a polypeptide that, like soluble TNFSF13b, induces cell proliferation, but unlike soluble TNFSF13b, is not neutralized by an antibody (4A5-3.1.1-B4) to TNFSF13b.

As indicated above, the TNFSF13b variants of the present invention will possess TNFSF13b B cell proliferation activity. TNFSF13b has been identified as a member of the TNF superfamily and has been shown to induce B cell proliferation and differentiation, both *in vivo* and *in vitro* [Moore *et al.*, *Science* 285:260-263 (1999)]. Indicators of TNFSF13b biological activity can be assessed by one or more of the several *in vitro* or *in vivo* assays known in the art. (see, for example, Moore, P.A., *et al.*, *Science*, 285:260-263 (1999); Schneider, P., *et al.*, *J. Exp. Med.*, 189:1747-1756 (1999); Shu, H., *et al.*, *J. Leuko. Biol.*, 65:680-683 (1999); Mukhopadhyay, A., *et al.*, *J. Biol. Chem.*, 274:15978-15981 (1999); Mackay, F. *et al.*, *J. Exp. Med.*, 190:1697-1710 (1999); and Gross, J.A., *et al.*, *Nature*, 404:995-999 (2000)). In addition, the functional activity of soluble TNFSF13b and the TNFSF13b variants of the present invention can be assayed using a cell proliferation assay as described in Example 4. This cell proliferation assay may also be used to determine the ability of antibodies to TNFSF13b to neutralize the activity of TNFSF13b variants described above.

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TNFSF13b has been found to be expressed in cells of monocytic lineage, kidney, lung, peripheral leukocyte, bone marrow, T cell lymphoma, B cell lymphoma, activated T cells, stomach cancer, smooth muscle, macrophages, and cord blood tissue (U.S. Patent 6,403,770). In one study, TNFSF13b mRNA was found to be predominantly expressed in peripheral blood leukocytes, spleen, lymph node and bone marrow, with detectable expression in placenta, heart, lung, fetal liver, thymus and pancreas [Moore *et al.*, *Science* 285:260-263 (1999)]. Analysis of a panel of cell lines demonstrated high expression of TNFSF13b in HL60 cells, detectable expression in K562, but no expression in Raji, HeLa, or MOLT-4 cells. It thus appears that TNFSF13b mRNA expression is enriched in the immune system. TNFSF13b also appears to be expressed highly in primary dendritic cells. Considering its gene expression and functional activity, the TNFSF13b protein may function in cellular signaling between B cells and monocytes, through which TNFSF13b may regulate B cell immune responses, as well as some T cell immune responses.

Like other members of the TNF family, TNFSF13b exhibits activity on leukocytes including, for example, monocytes, lymphocytes (e.g., B cells) and neutrophils. TNFSF13b is thereby active in directing the proliferation, differentiation and migration of these cell types. Such activity is useful for immune enhancement or suppression, myeloprotection, stem cell mobilization, acute and chronic inflammatory control and treatment of leukemia. Assays for measuring such activity are known in the art. For example, see Peters *et al.*, *Immun. Today* 17:273 (1996); Young *et al.*, *J. Exp. Med.* 182:1111 (1995); Caux *et al.*, *Nature* 390:258 (1992); and Santiago-Schwarz *et al.*, *Adv. Exp. Med. Biol.* 378:7 (1995).

Considering the cells and tissues where TNFSF13b is expressed as well as the activities modulated by TNFSF13b, a substantially altered (increased or decreased) level of expression of TNFSF13b in an individual compared to the standard or "normal" level is likely to render pathological conditions related to the bodily system(s) in which TNFSF13b is expressed and/or is active. Altered levels of TNFSF13b may be associated with a number of disorders of these tissues and cells, such as tumor and tumor metastasis, infection of bacteria, viruses and other parasites, immunodeficiencies (e.g., chronic variable immunodeficiency), septic shock, inflammation, cerebral malaria, activation of the HIV virus, graft-host rejection, bone resorption, rheumatoid arthritis, autoimmune diseases (e.g., rheumatoid arthritis and systemic lupus erythematosus) and cachexia.

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(wasting or malnutrition). Specifically, significantly higher or lower levels of TNFSF13b gene expression may be detected in certain tissues (e.g., bone marrow) or bodily fluids (e.g., serum, plasma, urine, synovial fluid or spinal fluid) taken from an individual having such a disorder, relative to a "standard" TNFSF13b gene expression level, i.e., the TNFSF13b expression level in tissue or bodily fluids from an individual not having the disorder.

Conditions or diseases caused by a decrease in the standard or normal level of TNFSF13b activity in an individual, particularly disorders of the immune system, can be treated by administration of TNFSF13b variants of the present invention. Thus, the invention provides a method of treatment of an individual in need of an increased level of TNFSF13b activity comprising administering to such an individual a pharmaceutical composition comprising an amount of an isolated TNFSF13b variant polypeptide of the invention, effective to increase the TNFSF13b activity level in such an individual.

An effective amount of an isolated TNFSF13b variant polypeptide of the invention may be therapeutically beneficial for any of a number of immune system disorders, including, but not limited to, an immunodeficiency (e.g., severe combined immunodeficiency (SCID)-X linked, SCID-autosomal, adenosine deaminase deficiency (ADA deficiency), X-linked agammaglobulinemia (XLA), Bruton's disease, congenital agammaglobulinemia, X-linked infantile agammaglobulinemia, acquired agammaglobulinemia, adult onset agammaglobulinemia, late-onset agammaglobulinemia, dysgammaglobulinemia, hypogammaglobulinemia, transient hypogammaglobulinemia of infancy, unspecified hypogammaglobulinemia, agammaglobulinemia, common variable immunodeficiency (CVID) (acquired), Wiskott-Aldrich Syndrome (WAS), X-linked immunodeficiency with hyper IgM, non X-linked immunodeficiency with hyper IgM, selective IgA deficiency, IgG subclass deficiency (with or without IgA deficiency), antibody deficiency with normal or elevated IgS, immunodeficiency with thymoma, Ig heavy chain deletions, kappa chain deficiency, B cell lymphoproliferative disorder (BLPD), selective IgM immunodeficiency, recessive agammaglobulinemia (Swiss type), reticular dysgenesis, neonatal neutropenia, severe congenital leukopenia, thymic alymphoplasia-aplasia or dysplasia with immunodeficiency, ataxia-telangiectasia, short limbed dwarfism, X-linked lymphoproliferative syndrome (XLP), Nezelof syndrome-combined immunodeficiency with IgS, purine nucleoside phosphorylase deficiency

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(PNP), MHC Class II deficiency (Bare Lymphocyte Syndrome) and severe combined immunodeficiency.) or conditions associated with an immunodeficiency.

TNFSF13b variant polypeptides of the present invention can be used in the treatment of infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, TNFSF13b variant polypeptides may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

### EXAMPLES

#### **Example 1: Expression and Purification of hTNFSF13b variant polypeptides polypeptides in *E. coli***

The bacterial expression vector pQE60 is used for bacterial expression in this example. (QIAGEN, Inc., Chatsworth, CA). pQE60 encodes ampicillin antibiotic resistance ("Ampr") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-triacetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., and suitable single restriction enzyme cleavage sites. These elements are arranged such that a DNA fragment encoding a polypeptide can be inserted in such a way as to produce that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide. However, a polypeptide coding sequence can optionally be inserted such that translation of the six His codons is prevented and, therefore, a polypeptide is produced with no 6 X His tag.

The nucleic acid sequence encoding the desired portion of a TNFSF13b variant polypeptide lacking the hydrophobic leader sequence is amplified from a cDNA clone using PCR oligonucleotide primers (based on nucleotide sequences encoding, e.g., variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9)

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which anneal to the amino terminal encoding DNA sequences of the desired portion of the TNFSF13b variant polypeptide-encoding nucleic acid and to sequences in the construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning, the 5' and 3' primers have nucleotides corresponding or complementary to a portion of the coding sequence of the TNFSF13b variant polypeptide-encoding nucleic acid, according to known method steps. One of ordinary skill in the art would appreciate, of course, that the point in a polynucleotide sequence where the 5' primer begins can be varied to amplify a desired portion of the complete polypeptide-encoding polynucleotide shorter or longer than the polynucleotide which encodes the mature form of the polypeptide.

The amplified nucleic acid fragments and the vector pQE60 are digested with appropriate restriction enzymes, and the digested DNAs are then ligated together. Insertion of the TNFSF13b variant polypeptide-encoding DNA into the restricted pQE60 vector places the TNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9 polypeptide coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating AUG codon. The associated stop codon prevents translation of the six histidine codons downstream of the insertion point.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook, *et al.*, 1989; Ausubel, 1987-1998. *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kanr"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing TNFSF13b variant polypeptides, is available commercially from QIAGEN, Inc. Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/mL) and kanamycin

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(25 µg/mL). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm (“OD600”) of between 0.4 and 0.6. Isopropyl-beta-D-thiogalactopyranoside (“IPTG”) is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for three to four hours. Cells then are harvested by centrifugation.

The cells are then stirred for three to four hours at 4 °C in 6 M guanidine hydrochloride, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the TNFSF13b variant polypeptide is dialyzed against 50 mM sodium acetate buffer, pH 6, supplemented with 200 mM sodium chloride. Alternatively, a polypeptide can be successfully refolded by dialyzing it against 500 mM sodium chloride, 20% glycerol, 25 mM Tris hydrochloride, pH 7.4, containing protease inhibitors.

If insoluble protein is generated, the protein is made soluble according to known method steps. After renaturation, the polypeptide is purified by ion exchange, hydrophobic interaction, and size exclusion chromatography. Alternatively, an affinity chromatography step such as an antibody column is used to obtain pure TNFSF13b variant polypeptide. The purified polypeptide is stored at 4 degrees C or frozen at negative 40 degrees C to negative 120 degrees C.

**Example 2: Cloning and Expression of hTNFSF13b variant polypeptides in a Baculovirus Expression System**

In this example, the plasmid shuttle vector pA2 GP is used to insert the cloned DNA encoding the mature TNFSF13b variant polypeptide into a baculovirus, using a baculovirus leader and standard methods as described in Summers, *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by the secretory signal peptide (leader) of the baculovirus gp67 polypeptide and convenient restriction sites such as BamHI, Xba I, and Asp718. The polyadenylation site of the simian virus 40 (“SV40”) is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation,

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followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that expresses the cloned polynucleotide.

Other baculovirus vectors are used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow, *et al.*, *Virology* 170:31-39 (1989).

The cDNA sequence encoding the mature TNFSF13b variant polypeptide in a clone, lacking the AUG initiation codon and the naturally associated nucleotide binding site, is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. Non-limiting examples include 5' and 3' primers having nucleotides corresponding or complementary to a portion of the coding sequence of a TNFSF13b variant polypeptide-encoding polynucleotide, according to known method steps.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit (e.g., "Geneclean," BIO 101 Inc., La Jolla, CA). The fragment is then digested with the appropriate restriction enzyme and again is purified on a 1% agarose gel. This fragment is designated herein "F1."

The plasmid is digested with the corresponding restriction enzymes and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, CA). This vector DNA is designated herein "V1."

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid bearing a human TNFSF13b variant polypeptide-encoding polynucleotide using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing a TNFSF13b

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variant polypeptide-encoding polynucleotide will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. The resulting plasmid is designated herein as pBacTNFSF13b variant.

Five µg of the plasmid pBacTNFSF13b variant plasmid construct is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold® baculovirus DNA", PharMingen, San Diego, CA), using the lipofection method described by Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA* 84: 7413-7 (1987). 1 µg of BaculoGold® virus DNA and 5 µg of the plasmid pBacTNFSF13b variant are mixed in a sterile well of a microtiter plate containing 50 µL of serum-free Grace's medium (Life Technologies, Inc., Rockville, MD). Afterwards, 10 µL Lipofectin plus 90 µL Grace's medium are added, mixed and incubated for fifteen minutes at room temperature. Then, the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 mL Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for five hours at 27 degrees C. After five hours, the transfection solution is removed from the plate and 1 mL of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27 degrees C for four days.

After four days, the supernatant is collected, and a plaque assay is performed. An agarose gel with "Blue Gal" (Life Technologies, Inc., Rockville, MD) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies, Inc., Rockville, MD, pp. 9-10). After appropriate incubation, blue stained plaques are picked with a micropipettor tip (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µL of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later, the supernatants of these culture dishes are harvested and then stored at 4 degrees C.

To verify the expression of the TNFSF13b variant polypeptide, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus at a multiplicity of infection ("MOI") of about

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two. Six hours later, the medium is removed and replaced with SF900 II medium minus methionine and cysteine (available, e.g., from Life Technologies, Inc., Rockville, MD). If radiolabeled polypeptides are desired, 42 hours later, 5 mCi of  $^{35}\text{S}$ -methionine and 5 mCi  $^{35}\text{S}$ -cysteine (available from Amersham, Piscataway, NJ) are added. The cells are further incubated for sixteen hours and then harvested by centrifugation. The polypeptides in the supernatant as well as the intracellular polypeptides are analyzed by SDS-PAGE, followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified polypeptide can be used to determine the amino terminal sequence of the mature polypeptide and, thus, the cleavage point and length of the secretory signal peptide.

**Example 3: Cloning and Expression of a TNFSF13b VARIANT Polypeptide in Mammalian Cells**

A typical mammalian expression vector contains at least one promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., RSV, HTLV, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pIRESpneo, pRetro-Off, pRetro-On, PLXSN, or pLNCX (Clontech Labs, Palo Alto, CA), pcDNA3.1 (+/-), pcDNA/Zeo (+/-) or pcDNA3.1/Hygro (+/-) (Invitrogen), PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Other suitable mammalian host cells include human Hela 293, H9, Jurkat cells, mouse NIH3T3, C127 cells, Cos 1, Cos 7 and CV 1, quail QC1-3 cells, mouse L cells, and Chinese hamster ovary (CHO) cells.

Alternatively, the gene is expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as

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DHFR (dihydrofolate reductase), GPT neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded polypeptide. The DHFR marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) [Murphy, *et al.*, *Biochem. J.* 227:277-9 (1991); Bebbington, *et al.*, *Bio/Technology* 10:169-75 (1992)]. Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of polypeptides.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus [Cullen, *et al.*, *Mol. Cell. Biol.* 5:438-47 (1985)] plus a fragment of the CMV-enhancer [Boshart, *et al.*, *Cell* 41:521-30 (1985)]. Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI, and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

#### **Example 3(a): Cloning and Expression in COS Cells**

The expression plasmid, pTNFSF13b variant HA, is made by cloning a cDNA encoding TNFSF13b variant polypeptide into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) or HIS tag (see, e.g., Ausubel, *supra*) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin polypeptide

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described by Wilson, *et al.*, *Cell* 37:767-8 (1984). The fusion of the HA tag to the target polypeptide allows easy detection and recovery of the recombinant polypeptide with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the TNFSF13b variant polypeptide is cloned into the polylinker region of the vector so that recombinant polypeptide expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The TNFSF13b variant polypeptide-encoding cDNA of a clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of TNFSF13b variant polypeptides in *E. coli*. Non-limiting examples of suitable primers include those based on the nucleotide sequences encoding variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9 polypeptide.

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with suitable restriction enzyme(s) and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, La Jolla, CA), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the TNFSF13b variant polypeptide-encoding fragment.

For expression of recombinant TNFSF13b-variant polypeptide, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook, *et al.*, *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of the TNFSF13b variant polypeptide-encoding polynucleotide by the vector.

Expression of the TNFSF13b variant polypeptide-HA fusion is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow, *et al.*, *Antibodies: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing  $^{35}$ S-cysteine for eight hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing

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RIPA buffer: 150 mM sodium chloride, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson, *et al.*, cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated polypeptides then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

#### Example 3(b): Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of the TNFSF13b variant polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary cells or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented [see, e.g., Alt, *et al.*, *J. Biol. Chem.* 253:1357-70 (1978); Hamlin and Ma, *Biochem. et Biophys. Acta* 1097:107-43 (1990); and Page and Sydenham, *Biotechnology* 9:64-8 (1991)]. Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach can be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus [Cullen, *et al.*, *Mol. Cell. Biol.* 5: 438-47 (1985)] plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) [Boshart, *et al.*, *Cell* 41: 521-30 (1985)]. Downstream of the promoter are BamHI, XbaI, and Asp718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites, the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human beta-actin promoter, the

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SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the TNFSF13b variant polypeptide in a regulated way in mammalian cells [Gossen, and Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547-51 (1992)]. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with restriction enzymes and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete the TNFSF13b variant polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. Non-limiting examples include 5' and 3' primers having nucleotides corresponding or complementary to a portion of the coding sequences of a TNFSF13b variant polypeptide-encoding polynucleotide, according to known method steps.

The amplified fragment is digested with suitable endonucleases and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary (CHO) cells lacking an active DHFR gene are used for transfection. Five µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSV2-neo using lipofectin. The plasmid pSV2-neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 µg/mL G418. After two days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/mL of methotrexate plus 1 µg/mL G418. After about ten to fourteen days, single clones are trypsinized and then seeded in six-well petri dishes or 10 mL flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM,

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800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new six-well plates containing even higher concentrations of methotrexate (1 mM, 2 mM, 5 mM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 to 200 mM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

#### **Example 4: Functional Activity of Anti-hTNFSF13b Human Antibodies**

Neutralizing activity of anti-hTNFSF13b human antibodies is measured using a murine IL-1 dependent B cell line, T1165.17. The cells are washed three times with assay media (RPMI1640 containing 10% FBS, 1 mM sodium pyruvate,  $5 \times 10^{-5}$  M 2-mercaptoethanol and penicillin, streptomycin and fungizone) to remove IL-1. The cells are resuspended at 100,000 cells/ml in assay media containing 2.5 ng/ml soluble huTNFSF13b and plated at 5000 cells/well in a 96 well plate and incubated at 37°C in 5% CO<sub>2</sub>. Supernatants from ELISA positive hybridomas are included at a 1:4 dilution. Forty-eight hours later, 20 µl of Promega CellTiter 96 Aqueous One Solution (Madison, WI) is added and the plate incubated for 5 more hours at 37°C in 5% CO<sub>2</sub>. Absorbance is read at A490, to measure proliferation. As a control, antibodies are added to IL-1 stimulated cells.

Neutralizing antibodies are tested for the ability to inhibit variants of TNFSF13b or control TNFSF13b augmented primary human B cell proliferation in response to anti-IgM stimulation. Primary human B cells are isolated from human blood using CD19 positive selection using the MACS magnetic isolation system (Miltenyi Biotec, Auburn, CA). The B cells are added to wells of a 96-well plate at  $2 \times 10^5$  cells per well in complete RPMI containing 10% FCS (complete RPMI is RPMI1640 containing 10 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and  $1 \times 10^{-5}$  M β-mercaptoethanol). Some of the wells are coated with 10 µg/ml anti-human IgM in PBS (BD PharMingen, Clone G20-127), overnight at 4°C and washed four times with PBS before use. Some of the cells are stimulated with TNFSF13b or control, soluble hTNFSF13b (25 ng/ml) in the presence or absence of neutralizing anti-hTNFSF13b antibody (2.5 µg/ml).

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**Example 5: Species Crossreactivity of Anti-hTNFSF13b Human Antibodies with non-human TNFSF13b**

To determine the species crossreactivity of neutralizing mAbs, an ELISA was set up utilizing an antibody to hTNFSF13b, 4A5-3.1.1-B4 (disclosed in WO 03/016468), as both the capture and detecting mAb. Human recombinant TNFSF13b was used as the standard curve. Human TNFSF13b was detected in the culture supernatant from CHO cells transfected with a vector expressing hTNFSF13b, supernatants from cultured human monocytes or human serum or plasma. Supernatants from CHO cells expressing murine TNFSF13b were tested for reactivity in the ELISA and were negative. 4A5-3.1.1-B4 was also unable to immunoprecipitate murine TNFSF13b but was able to immunoprecipitate human TNFSF13b. Human TNFSF13b and murine TNFSF13b were used in the proliferation assay described in Example 4. Using this proliferation assay, 4A5-3.1.1-B4 was able to neutralize the proliferation induced by human TNFSF13b, but was unable to neutralize the proliferation induced by murine TNFSF13b. This result indicates that 4A5-3.1.1-B4 is unable to recognize murine TNFSF13b.

**Example 6: Identification of the Epitope for 4A5-3.1.1-B4**

The epitope to which antibody 4A5-3.1.1-B4 bound and neutralized human TNFSF13b was determined. Human and murine TNFSF13b sequences were aligned as shown below:

Mouse TNFSF13b	1 AFQGPETEQ	DVDLSAPPAP	CLPGCRHSQH	DDNGMNLRNI	IQDCLQLIA	49	
Human TNFSF13b	1 AVQGPEE---	-----	-----	-----	TV	TQDCLQLIA	18
Mouse TNFSF13b	50 DSDTPTIRKG	TYTFPVWLLS	FKRGNALEEK	ENKIVVRQTG	YFFIYSQVLY	99	
Human TNFSF13b	19 DSETPTIQKG	SYTFPVWLLS	FKRGSALEEK	ENKILVKETG	YFFIYGQVLY	68	
Mouse TNFSF13b	100 TDPIFAMGHV	IQRKKVHVFG	DESLVTLFR	CIQNMPKTLP	NNSCYSAGIA	149	
Human TNFSF13b	69 TDKYAMGHL	IQRKKVHVFG	DESLVTLFR	CIQNMPETLP	NNSCYSAGIA	118	
Mouse TNFSF13b	150 RLEEGDEIQL	AIPRENAQIS	RNGDDTFFGA	LKLL	183		
Human TNFSF13b	119 KLEEGDELQL	AIPRENAQIS	LDGDVTFFGA	LKLL	152		

A homology model was created for human TNFSF13b based on the known crystal structure for several TNF family members. Exposed residues that are different between mouse and human TNFSF13b are potential binding sites for 4A5-3.1.1-B4 since 4A5-3.1.1-B4 neutralizes human but not mouse TNFSF13b.

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Three potential epitopes were identified: 1) K71, T72, Y73, E105; 2) Q26, S29, L139, D140; and 3) L53, K55, E56, K119. The first epitope, K71, T72, Y73, E105, may additionally contain one or more of amino acids T69, T106, L107, and N109.

Mutagenesis was performed to make chimeric molecules by changing the amino acid sequence from human to mouse. Chimera A was L139R, D140N; Chimera B was K71P, T72I, Y73F; Chimera C was K71P, T72I, Y73F, E105K; Chimera D was L53V, K55R, E56Q; Chimera E was E105K.

Using the proliferation assay as described in Example 4, all of the chimeras were tested for functional activity and neutralization by 4A5-3.1.1-B4. Initial assays were performed using supernatants from 293 transient transfections for each of the chimeras and both human TNFSF13b and murine TNFSF13b parent molecules. All of the chimeras induced similar proliferation indicating that the chimeras produced were functional. Using 6 ug/ml of 4A5-3.1.1-B4, 100% neutralization was observed with human TNFSF13b and Chimeras A, B, D and E. No neutralization was observed for murine TNFSF13b or Chimera C. Purified TNFSF13b mutants were produced for Chimeras A, B, and C and the assay was repeated using 11 ng/ml of each parent TNFSF13b or chimera TNFSF13b and 1 ug/ml of 4A5-3.1.1-B4. The results showed 100% neutralization was observed with human TNFSF13b and Chimera A, 88% neutralization with Chimera B, and no neutralization was observed for murine TNFSF13b or Chimera C.

#### **Example 7: In vivo studies using transgenic mice and 4A5-3.1.1-B4**

Transgenic mice overexpressing soluble human TNFSF13b or TNFSF13b variants of the present invention are generated using established techniques as described by Hogan, B. et al. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory, NY] as modified by Fox and Solter (Mol. Cell. Biol. 8: 5470, 1988). Briefly, a DNA fragment encompassing the hTNFSF13b gene is microinjected into the male pronuclei of newly fertilized one-cell-stage embryos (zygotes) of the FVB/N strain. The embryos are cultured in vitro overnight to allow development to the two-cell-stage. Two-cell embryos are then transplanted into the oviducts of pseudopregnant CD-1 strain mice to allow development to term. To test for the presence of the transgene in the newborn mice, a small piece of toe is removed from each animal

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and digested with proteinase K to release the nucleic acids. A sample of the toe extract is subsequently subjected to PCR analysis to identify transgene-containing mice.

Human TNFSF13b transgenic mice had a dramatic increase in peripheral B cells, generally about three fold compared to age and sex matched littermates. There was a slight increase in peripheral T cells as well. The hTNFSF13b transgenic mice were treated with 4A5-3.1.1-B4 (antibody to hTNFSF13b) to determine if neutralization of hTNFSF13b would result in a reduction in B cell numbers back to normal levels. At 15 weeks old, female hTNFSF13b mice were injected subcutaneously twice a week for three weeks with either 25 ug of 4A5-3.1.1-B4 or isotype control antibody. Four days after the last injection of antibody, the mice were sacrificed and the spleen removed for analysis. B and T cell numbers were calculated by determining the percentage of CD19+ cells, for B cells, and CD3+ cells, for T cells using flow cytometry and absolute white blood cell count for each spleen. The results shown below in Table 2 demonstrate that in vivo administration of 4A5-3.1.1-B4 to hTNFSF13b transgenic mice is able to restore the normal numbers of T and B cells (average  $\pm$  standard deviation).

Table 2

Treatment Group	B cells ( $\times 10^6$ )	T cells ( $\times 10^6$ )
Wild type littermates	29 $\pm$ 11	46 $\pm$ 15
Transgenic + Isotype mAb	122 $\pm$ 30	75 $\pm$ 14
Transgenic + 4A5 mAb	29 $\pm$ 5	46 $\pm$ 12

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WE CLAIM:

1. Isolated nucleic acid comprising DNA having at least 95% sequence identity to a polynucleotide selected from the group consisting of:
  - (a) a polynucleotide encoding a polypeptide having the amino acid sequence of hTNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9; and
  - (b) a polynucleotide fragment of a polynucleotide as in (a).
2. An isolated polypeptide comprising a sequence of amino acid residues of hTNFSF13b variant 1, variant 2, variant 3, variant 4, variant 5, variant 6, variant 7, variant 8, or variant 9; and  
fragments thereof possessing TNFSF13b B cell proliferation activity.
3. A chimeric molecule comprising a hTNFSF13b variant polypeptide fused to a heterologous amino acid sequence.
4. The chimeric molecule of Claim 3, wherein said heterologous amino acid sequence is an epitope tag sequence.
5. The chimeric molecule of Claim 4, wherein said heterologous amino acid sequence is an Fc region of an immunoglobulin.
6. An antibody which neutralizes hTNFSF13b variant 8, variant 9, or variants 8 and 9.
7. The antibody of Claim 6, wherein said antibody is a monoclonal antibody.
8. The antibody of Claim 7, wherein said antibody is selected from the group consisting of a humanized antibody and a human antibody.

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9. A composition comprising a therapeutically effective amount of an active agent selected from the group consisting of:

- (a) a hTNFSF13b variant polypeptide of claim 2; and
- (b) an anti-hTNFSF13b variant antibody of claim 6.

10. A method of treating a mammal suffering from a disease, condition, or disorder associated with aberrant levels of a hTNFSF13b variant polypeptide comprising administering a therapeutically effective amount of a hTNFSF13b variant polypeptide of claim 2.

11. Use of a hTNFSF13b variant polypeptide in the manufacture of a medicament for the treatment of a disease, condition, or disorder associated with aberrant levels of a hTNFSF13b polypeptide.

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